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14. ABSTRACT **Enter a brief (approximately 200 words) unclassified summary of the most significant findings during the research period.**
Helicobacter pylori (*Hp*) is linked to chronic gastritis, peptic ulcer disease (PUD) and gastric cancer (GC), but it is unclear why infected individuals develop different diseases. GC annually claims 700,000 lives worldwide (seer.cancer.gov). Early detection of GC is crucial in improving prognosis, but biomarkers of disease are lacking. Our objective is to characterize unique genomic features of GC *Hp* isolates and gastric epithelial responses elicited by these isolates that could represent candidate biomarkers. We used novel human gastroids infected with a panel of *Hp* isolates from different gastric diseases. Infections with different *Hp* isolates revealed by real time PCR distinct expression of genes related to immunity, NOTCH signaling, metaplasia, cell survival and cell death. Isolates from GC and PUD elicited more pronounced changes at the mRNA level compared to uninfected cells. For example, gastroids infected with a GC *Hp* isolate induced mucin 2, an intestinal mucin, rather than mucin 5AC characteristic of gastric surface cells, which represents neoplastic change in the form of intestinal metaplasia. Flow cytometry confirmed similar changes in protein expression. We noted high expression of immunoregulatory proteins as well as NOTCH receptors and ligands in cells infected with a GC *Hp* isolate. Thus, additional studies with multiple *Hp* isolates leading to epithelial responses unique to them and deep genomic sequencing may reveal candidate biomarkers and understanding of potential targets for vaccine/therapy, respectively.

15. SUBJECT TERMS
Helicobacter pylori (Hp), gastritis, peptic ulcer disease (PUD), gastric cancer (GC), gastric disease, gastroids, organoids, biomarkers

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1. INTRODUCTION:

Helicobacter pylori (*Hp*) contributes to chronic gastritis, peptic ulcer disease (PUD) and gastric cancer (GC), but it is not clear why different individuals develop different gastroduodenal diseases. Our objectives are (**1**) to determine genetic features present in *Hp* GC isolates not present in those from PUD or gastritis, and (**2**) to determine in human gastric organoids if GC isolates, compared to non-GC isolates, induce expression of proteins that may represent candidate biomarkers of disease.

2. KEYWORDS:

Helicobacter pylori (Hp), gastritis, peptic ulcer disease (PUD), gastric cancer (GC), gastric disease, gastroids, organoids, biomarkers

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major Task 1: Determine by deep sequencing and comparative genomic analysis of *Hp* isolates from GC, PUD and gastritis cases genomic features in *Hp* GC isolates not present in those from PUD or gastritis. This task has 3 subtasks of which Subtask 1 was to submit IRB protocol for approval by institutional IRB (within first three months). Accomplished locally (approval date 6/21/17) and waiting on final approval at collaborator's site. Subtask 2. Culture and DNA extraction of collections of *Hp* strains from gastric cancer (GC), peptic ulcer disease (PUD) and gastritis (minimum of 10/group). Subtask 3. Perform comparative genomic analysis of *Hp* strains isolated from different gastroduodenal diseases whose DNA has been extracted. Subtasks 2 and 3 are in progress while analyzing results from Major Task 3. We still expect to accomplish the milestones within the planned timeframe of the SOW.
Major Task 2: Determine whether the genomic features found in cultured isolates are consistent with those present in *Hp* within biopsies in the context of influences by the host and other microbial communities. In progress.
Major Task 3: Determine whether infection of human gastric organoids by *Hp* GC isolates, in comparison to *Hp* non-GC isolates, results in differential epithelial expression of proteins that may represent candidate biomarkers of disease. While the first two tasks are in progress, this major task is one that is significantly advanced using some of the *Hp* isolates collected from different disease states.
Subtask 1. Expand at least three of the available human gastric organoid cultures. This was planned for months 9-12 and was 100% completed within the first 6 months and the organoids are in use for subtasks 2 and 3, below. Currently we have grown 7 different gastric organoids.
Subtask 2. Infect each of the human gastric organoid cultures with five *Hp* strains from each of the gastroduodenal diseases (GC, PUD and gastritis) and assess levels of PD-L1, B7-H3 and B7-H4 at the protein (flow cytometry) and mRNA (real time PCR) levels. This was work planned for Year 2, but we moved it ahead and have made some important findings. This is about 30-40% completed.
Subtask 3. Determine whether soluble forms of PD-L1, B7-H3 and/or B7-H4 are present in supernatants of infected cultures and compare the levels of expression induced by *Hp* strains from different disease states. In Progress. Supernatants have been collected for analysis after we analyze data from subtask 2.

What was accomplished under these goals?

During the current reporting period, we submitted IRB protocols at two different institutes to secure access to clinical samples for isolation of *Hp*. While that process was underway we successfully established a panel of human gastric organoid (gastroids) cultures (See **Appendix Table 1, Figures 1-3**) and used them to implement an *in vitro* model of infection with a panel of *Hp* isolates from the different gastric diseases (gastritis, PUD and GC). The model of infection requires culture of gastroids on tissue culture inserts with collagen that leads to the gastroids opening from their spheroid shape into polarized monolayers. Polarized monolayers (**Appendix Figure 4**) allow infection at the apical surface, as occurs *in vivo*. Infections with different *Hp* isolates from gastritis, PUD and GC revealed by real time PCR distinct responses in genes related to immune regulation, NOTCH signaling, metaplasia, cell survival and cell death. As we hypothesized, isolates from the more serious diseases elicited the more pronounced changes at the mRNA level (**Appendix Tables 2 and 3)** when compared to uninfected cells. For instance, mucus which is vital for the defense of the gastrointestinal tract is different in the stomach and intestine. Mucin 2, an intestinal mucin, rather than gastric mucin 5AC, was highly induced in gastroids by the GC *Hp* isolate and this is a sign of intestinal metaplasia. Similar results were obtained with a panel of established cell lines, which included HS738, a non-transformed gastric cell line (data not shown). In flow cytometry studies, to confirm that the observations at the mRNA level also resulted in elevated protein expression, we noted that expression of immunoregulatory proteins as well as NOTCH receptors and ligands were highest in cells infected with the GC *Hp* isolate, CA8 (**Appendix Figure 5**). Ongoing studies include panels of additional *Hp* isolates from the different gastric diseases, of which we have an ample panel. Also, current studies are investigating how the noted responses influence the T cell repertoire by adding co-culturing T cells with the gastroids infected with the different strains. Multiple reports have shown increased T regulatory cells in tumors, including GC.

Although there has been a delay, due to the lengthy process in securing IRB approval to obtain biopsies from different *Hp*-infected subjects for use of freshly isolated *Hp* and deep sequencing, we will be able to obtain the needed number of samples soon and we have also initiated collaboration with a lab that routinely isolate *Hp* and we will have a student from that lab join us. The deep sequencing studies will proceed quickly once we have all the planned samples.

What opportunities for training and professional development has the project provided?

The PI, a postdoc and a medical student have developed new skills as a result of this project. The PI implemented novel human organoid technology via collaboration with Dr. Mary Estes (Baylor College of Medicine) and her lab, via one-on-one visits, has guided Dr. Alex Peniche with valuable tips on the growth of the organoids. Also, former lab members using this technology have helped implement it. Also, a second year medical student spent the summer in the lab and learned to grow human cells in tissue culture inserts, Hp culture, infections of human cells, real-time PCR and staining for flow cytometry. Some of the findings were presented in the institutional summer research symposium in which the student received the first place award for best poster in Infection and Immunity.

How were the results disseminated to communities of interest?

The results have only been partially disseminated in the institution as they are not ready for broad dissemination at national conferences or symposia.

What do you plan to do during the next reporting period to accomplish the goals?

We have our local IRB approval and anticipate final approval of the IRB at our collaborator's institution, which will allow us to proceed fully with freshly isolated strains of *Hp* for infections of the human gastric organoids, confirm that isolates from each disease state induce responses that are different depending on the disease, but consistent for all isolates from the same gastric disease. Then, by deep sequencing we expect to identify genes or sequences in GC-derived strains that are conserved among them, but not present in strains isolated from PUD or gastritis cases. We also expect to determine whether any genetic features identified in *Hp* strains from GC cases in Aim 1, which had been previously maintained in culture, reflect the features that are present *in vivo* in the context of host or local microbiome influences. Finally, we anticipate to have identified candidate biomarkers of disease.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

Our findings with the primary cultures of human gastric organoids infected with *Hp* bacteria isolated from patients with gastritis, peptic ulcer disease and gastric cancer are showing that *Hp* isolates from gastric cancer cases lead to epithelial responses associated with the process of carcinogenesis, although preliminary, are provocative. The studies will be followed in the second year of funding with examination of the fluid phase of the cultures for the identification of candidate biomarkers of disease, which will be important in early detection and in monitoring. Genomic sequencing of *Hp* isolates from gastric cancer cases compared to non-cancer cases is expected to provide insights into why some people who are infected develop

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

There is nothing to report at this time, since the findings are still preliminary and additional studies are needed.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

There are no significant planned changes from the original proposal.

Actual or anticipated problems or delays and actions or plans to resolve them.

The only delay has been in obtaining the fresh clinical samples to isolate *Hp* to compare against isolates that have been cultured previously. Our internal protocol had to be modified and is tentatively approved while the IRB protocol at our collaborator's institution has been going back and forth, but the last set of questions were easy to address and we anticipate final approval soon. Nonetheless, we have been approached by a lab that routinely isolates *Hp* for deep sequencing and they have a significant collection ready and can provide well-identified isolates.

Changes that had a significant impact on expenditures

There are no significant changes.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

In Progress. Final approval expected soon.

Significant changes in use or care of vertebrate animals

Not Applicable.

Significant changes in use of biohazards and/or select agents

Not Applicable.

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications.

Nothing to Report.

Books or other non-periodical, one-time publications.

Nothing to Report.

Other publications, conference papers and presentations.

Nothing to Report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Not Applicable.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report during the first year of funding.

- **Inventions, patent applications, and/or licenses**

Nothing to Report.

- **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Victor E. Reyes, PhD
Project Role: Principal Investigator
Researcher Identifier: 034470
Nearest person month worked: 2.4
Contribution to Project: Project leader, experimental design, data interpretation, communications
Funding Support: N/A

Name: Yuriy Fofanov, PhD
Project Role: Coinvestigator
Researcher Identifier: 241077
Nearest person month worked: 0.48
Contribution to Project: He oversees the analysis and interpretation of next generation DNA sequencing (NGS) data. He is consulted on selection of methods to analyze genomic data.
Funding Support: N/A

Name: Iryna V. Pinchuk, PhD - 192921
Project Role: Coinvestigator
Researcher Identifier: 192921
Nearest person month worked: 0.6
Contribution to Project: She has been responsible for developing the local IRB protocol and the IRB protocol by Dr. Suarez-Gould at Baylor in conjunction with Dr. Powell. She maintains communications from the IRB and Dr. Gould regarding the protocols. Her expertise in the isolation of the mucosal cells from GI human mucosa is needed as part of the studies and she has helped with the training of the postdoctoral fellow.
Funding Support: N/A

Name: Don W. Powell, MD
Project Role: Coinvestigator
Researcher Identifier: 050842
Nearest person month worked: 0.12
Contribution to Project: As a clinician and Director of the Division of Gastroenterology, he has been consulted during the IRB protocol development and revisions.
Funding Support: N/A

Name: Levent Albayrak
Project Role: Programmer
Researcher Identifier: 241231
Nearest person month worked: 1.2
Contribution to Project: He is tasked with development of computational tools to quickly and efficiently identify highly specific and robust signatures essential for this research.
Funding Support: N/A

Name: George Golovko
Project Role: Research Scientist
Researcher Identifier: 241207
Nearest person month worked: 1.2
Contribution to Project: He is responsible for developing new bioinformatics functions/modules and modifying existing ones or implementing new pipelines to perform analysis. He will also participate in the collection of new software tools, and participate in the bioinformatic analysis of the sequencing data.
Funding Support: N/A

Name: Kamil Khanipov
Project Role: Research Technician
Researcher Identifier: 241236
Nearest person month worked: 1.2
Contribution to Project: He is responsible for management, filtering and preparation of data for downstream analysis as well as testing and debugging the tools.
Funding Support: N/A

Name: Alex-Giovanny Peniche-Trujillo, PhD
Project Role: Postdoctoral Fellow
Researcher Identifier: 236476
Nearest person month worked: 12
Contribution to Project: Day to day performance of experiments, maintenance of bacterial and cell cultures, procurement of reagents.
Funding Support: N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Organization Name: Baylor College of Medicine (Milena Gould Suarez, MD)
Location of Organization: Houston, TX
Partner's contribution to the project: Dr. Gould is an Assistant Professor of Medicine in the department of Medicine, Section of Gastroenterology & Hepatology. She is the Medical Director of the Gastroenterology clinic at Smith Clinic as part of Harris Health Services. She has been responsible for the development of the IRB protocol at her institution in order to recruit from among the patients that she sees for the biopsy specimens to be used to freshly isolate *Hp*.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Table 1. Panel of human gastric organoid (gastroids) cultures stablished

Name	Age	Gender	Ethnic group	Surgery type	location
G1	25	M	Caucasian	Biopsy	antrum
G2	43	F	N/A	Biopsy	antrum
G3	63	F	Asian	Biopsy	antrum
G4	40	F	African American	Biopsy	antrum
G7	75	F	Caucasian	Biopsy	Upper part
G102	55	M	Caucasian	Biopsy	Body
G104	64	F	Caucasian	Biopsy	antrum

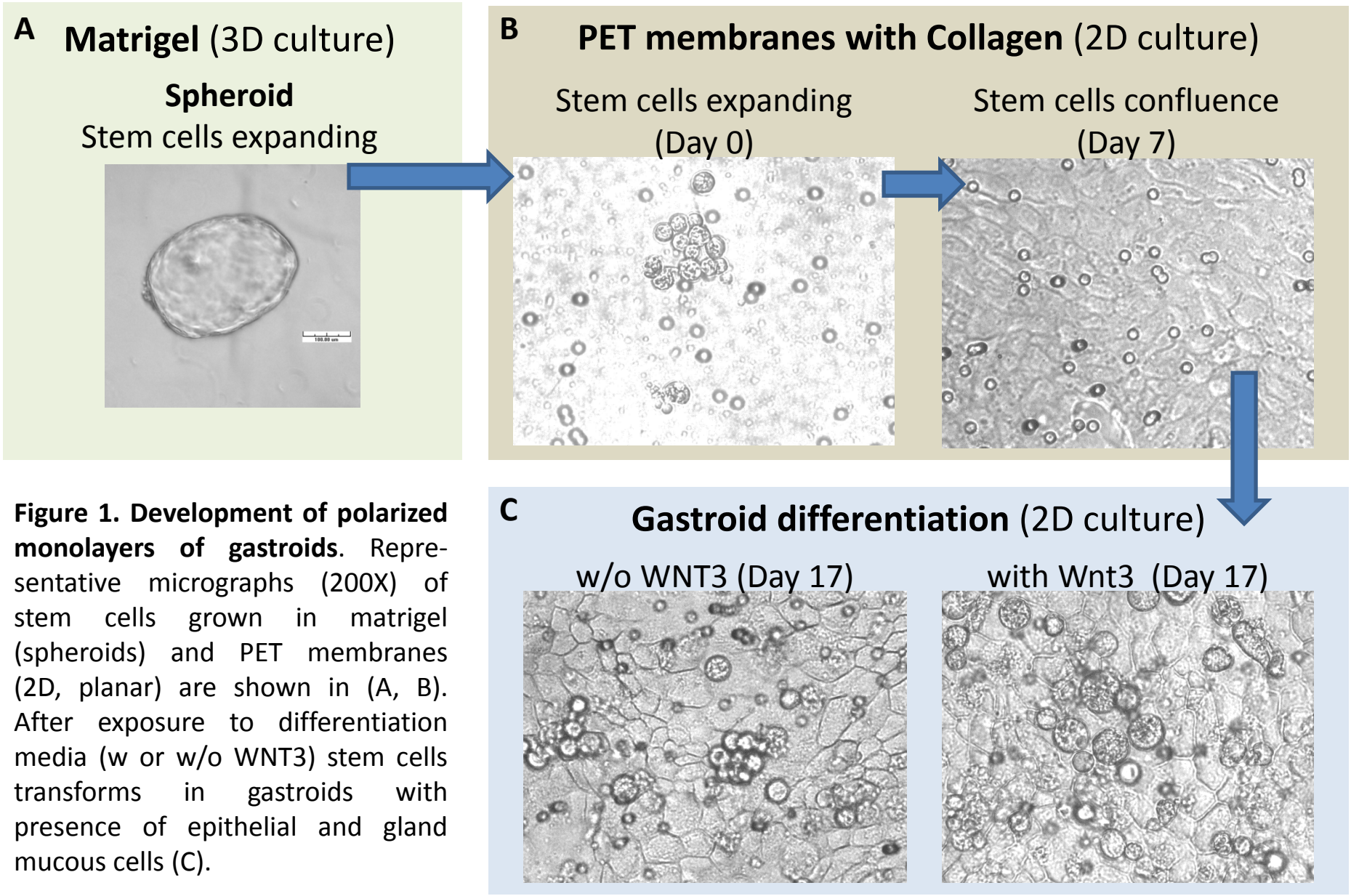
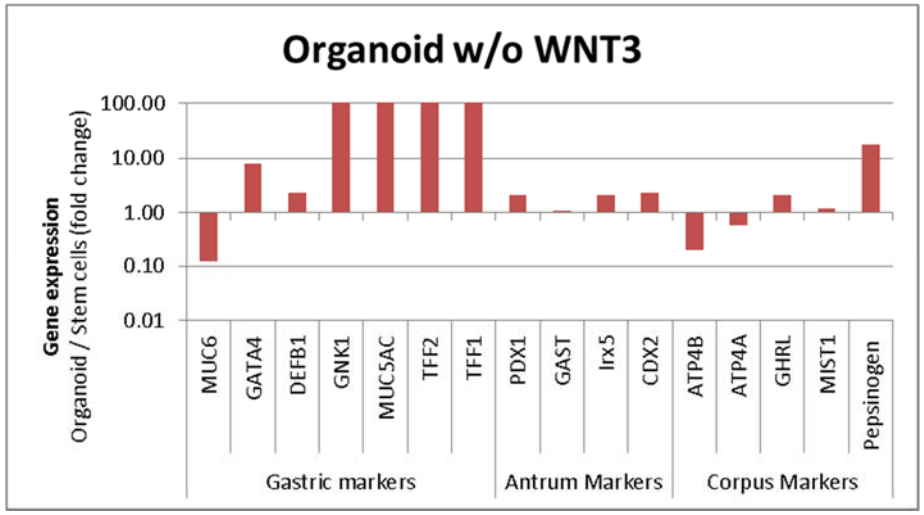
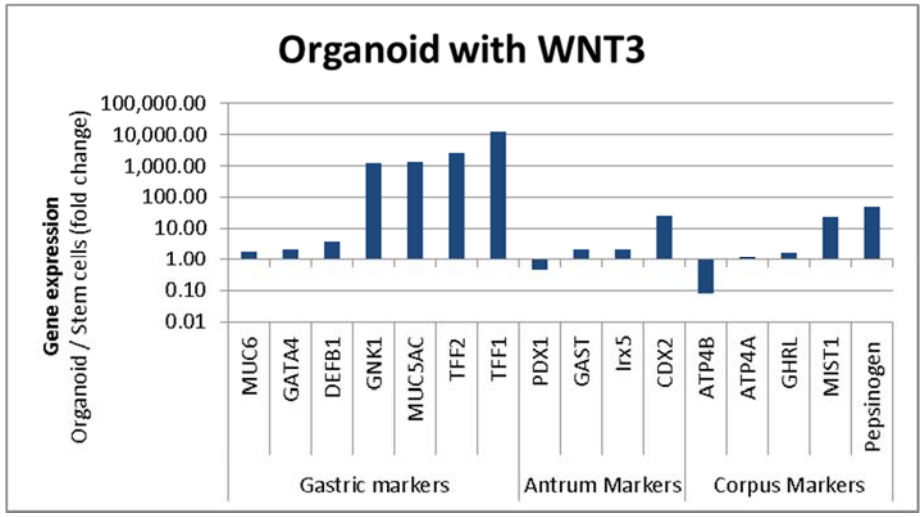


Figure 1. Development of polarized monolayers of gastroids. Representative micrographs (200X) of stem cells grown in matrigel (spheroids) and PET membranes (2D, planar) are shown in (A, B). After exposure to differentiation media (w or w/o WNT3) stem cells transforms in gastroids with presence of epithelial and gland mucous cells (C).

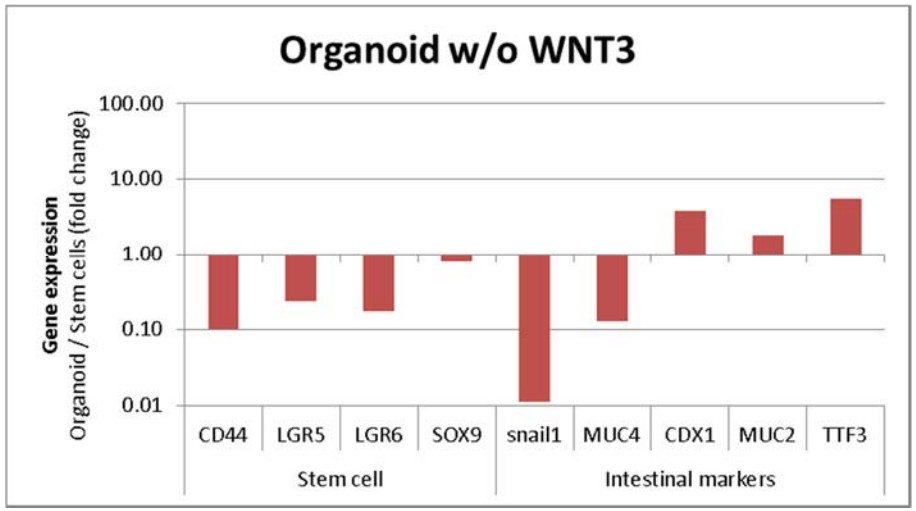


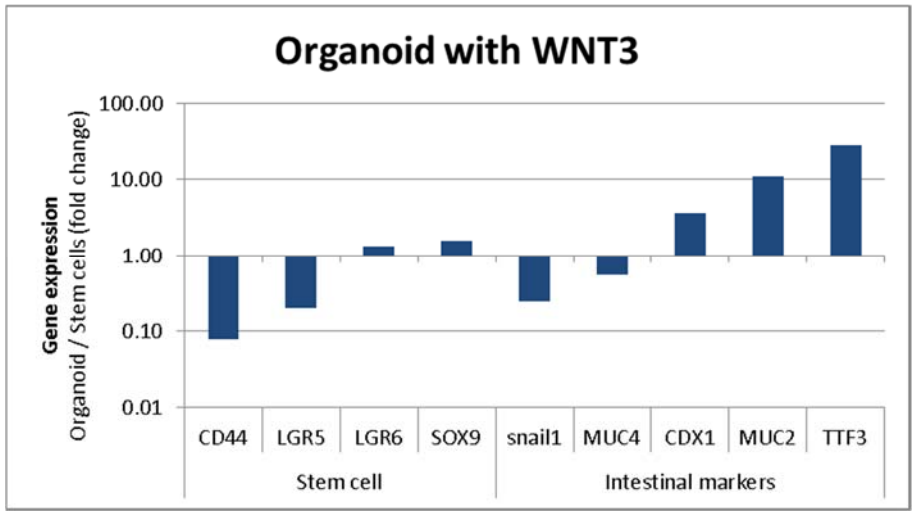


Gene	Stem Cells	Organoid w/o WNT3	Organoid WNT3
Gastric markers			
MUC6	2.0	0.2	3.5
GATA4	5.7	46.0	12.8
DEFB1	0.4	1.0	1.8
GNK1	17.8	40,623	21,694
MUC5AC	0.1	358.3	144.0
TFF1	0.6	3,397	6,985
TFF2	0.4	337.8	948.8
Antrum markers			
PDX1	3.2	1.2	388.0
GAST	0.6	0.6	1.3
Irx5	0.6	1.3	1.4
CDX2	0.1	0.2	2.4
Corpus markers			
ATP4A	2.2	1.3	2.8
ATP4B	7.6	1.5	0.6
GHRL	0.9	1.9	1.5
MIST1	1.3	1.6	32.1
Pepsinogen	1.9	34.1	95.3

<2	1	>2

Figure 2. Differentiation protocol promoted development of gastric markers. Gene expression analysis determine the increase of gastric function markers (Mucin 5AC, Defensin β1, and TTF1), as well the presence of genes associated with the antrum and corpus regions of stomach.





Gene expression of Stem and intestinal markers

Gene	Stem Cells	Organoid w/o WNT3	Organoid WNT3
Loss of stem cell markers			
CD44	13.1	1.3	1.0
LGR5	4.2	1.0	0.8
LGR6	2.5	0.4	3.3
SOX9	4.0	3.2	6.3
Intestinal markers			
snail1	123.6	1.4	30.1
MUC2	0.7	1.3	7.9
MUC4	7.0	0.9	3.8
CDX1	0.8	2.9	2.7
TTF3	19.6	107.6	550.7

<2	1	>2

Figure 3. Reduction of gastric Stem cell lineage after differentiation protocol. Gene expression analysis was made comparing stem cell and gastroids, in order to confirm in the later, loss of stem cells markers (CD44, LGR5, LGR6, and SOX9), as well the acquisition of undesired intestinal markers (MUC2, CDX1, TTF3) under the influence or not of WNT3.

Confocal images of gastroids in 2D culture (PET membrane insert)

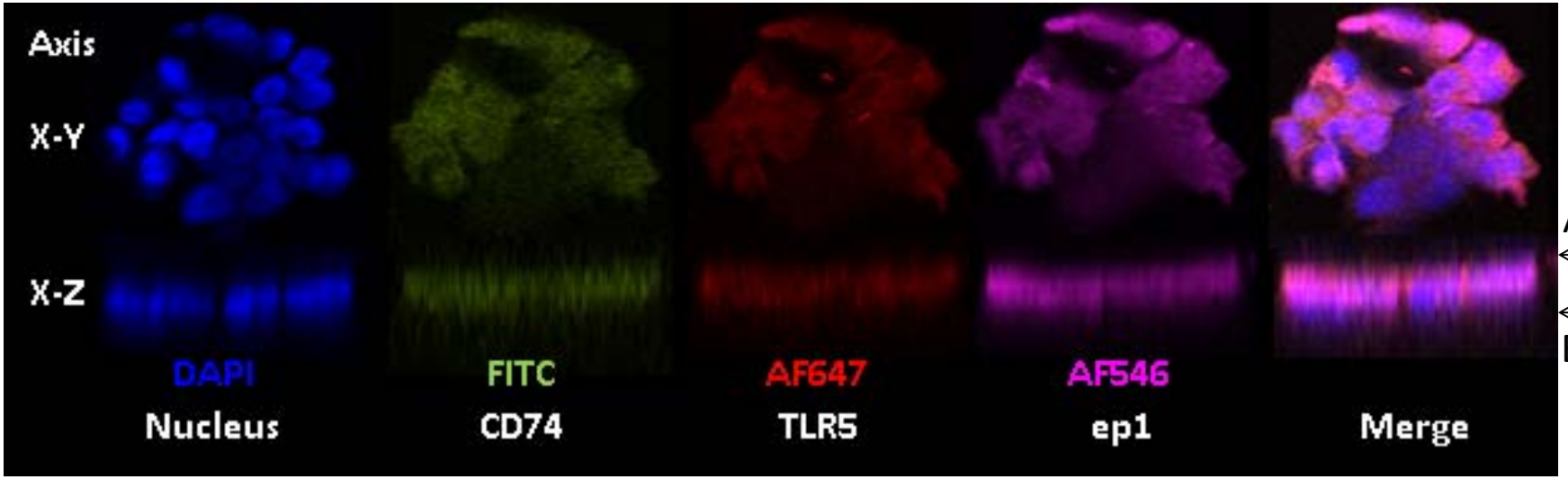


Figure 4. Development of epithelial polarized gastroids cultures. Confocal images (top) and gene expression analyses (bottom) confirm the presence of markers of cell polarization (between apical and basal areas) in gastroid cultures.

Gene expression of polarized epithelial cells

Gene	Stem Cells	Organoid w/o WNT3	Organoid WNT3
Polarized epithelium			
CD74	11.6	16.9	7.9
TLR4	0.6	1.1	0.7
TLR5	0.7	3.1	1.6
E-cadherin	35.3	459.8	626.0
ep1	1.0	11.8	95.3

<2	1	>2

Table 2. Gene expression of metaplasia, cell survival and cell death factors after *Hp* infection

Gene	Organoid w/o WNT3			Organoid WNT3		
H.pylory strain	51B	LC11	CA8	51B	LC11	CA8
Protective factors						
TFF1	1.2	2.0	1.9	0.8	0.8	0.9
TFF2	1.7	2.7	1.1	0.6	1.2	1.2
TTF3	2.3	2.7	0.7	1.9	1.4	3.5
MUC1	0.6	0.0	0.7	0.6	1.6	3.6
MUC5AC	0.4	1.3	1.1	0.6	0.1	0.4
Metaplasia Markers						
Gastrin	2.1	2.7	1.1	0.7	0.6	0.9
Pepsinogen	1.4	4.0	1.5	0.2	0.2	0.2
MUC2	0.5	6.0	8.3	1.1	0.3	0.1
MUC4	2.4	11.7	1.4	0.4	1.0	0.5
MUC6	52.2	30.5	10.3	15.7	1.5	0.2
CDX1	0.5	1.5	0.6	0.7	2.2	1.7
CDX2	5.1	11.2	2.0	0.4	0.4	0.4
snail1	8.7	10.7	6.5	0.2	1.4	0.1
PDX1	0.7	0.6	0.2	0.9	0.8	4.1

<2	1	>2

Gene	Organoid w/o WNT3			Organoid WNT3		
H.pylory strain	51B	LC11	CA8	51B	LC11	CA8
. Cell survival						
AEG-1 (astrocy	0.7	0.7	1.0	0.7	0.5	0.6
CEBPB	4.7	8.1	1.6	1.3	0.3	0.4
CyclinD1	1.1	1.3	0.9	0.7	0.4	0.8
HIF1a	2.6	2.7	3.7	0.7	0.7	0.8
Cmyc	16.8	6.5	1.9	0.9	0.6	0.4
C-myc	0.3	1.3	1.0	0.3	0.4	0.2
COX2	35.5	0.9	1.7	0.6	0.1	0.3
HIVEP3	0.2	0.1	0.9	1.7	0.3	2.6
Nanog	5.6	2.4	3.0	1.5	1.2	1.2
Oct3/4	1.3	0.7	1.3	1.0	0.4	0.4
. Necroptosis						
Ki67	15.6	70.8	8.3	0.6	0.2	0.6
Bmf	0.8	8.3	1.3	0.7	0.1	0.8
Bnip3	2.0	19.0	1.0	0.3	0.3	0.5
. Apoptosis						
BAX	0.4	0.1	1.4	1.6	0.2	0.5
p53	0.4	0.2	0.5	0.2	1.7	0.6
p21	1.7	0.6	2.1	0.6	1.7	3.4
Caspase3	0.6	0.7	0.8	0.7	2.7	2.1
.Anti-apoptosis						
BCL2	1.1	4.6	1.5	1.2	29.3	0.6
Irx5	2.2	51.1	3.5	0.8	0.4	3.8

<2	1	>2

Infections with different *Hp* isolates from gastritis (51B), PUD (LC11) and GC (CA8) revealed by real time PCR distinct responses in genes related to immune regulation

Table 3. Gene expression of inmmunoregulatory factors and NOTCH signaling after *Hp* infection

Gene	Organoid w/o WNT3			Organoid WNT3			N87-NCI (gastric epithelial cells)		
H.pylory strain	51B	LC11	CA8	51B	LC11	CA8	51B	LC11	CA8
Immunoregulatory factors									
B7-H1	5.2	59.9	6.5	0.3	0.2	0.1	0.8	2.7	2.1
B7-H2	2.6	1.4	1.5	0.0	0.0	0.0	1.2	1.1	0.9
B7-H3	0.7	0.5	0.6	0.5	0.4	0.4	0.4	0.7	0.5
B7-H4	0.8	0.7	1.3	0.2	0.4	0.7	0.8	2.7	2.1
NOTCH signaling									
DLL1	20.2	8.4	52.5	5.2	1.5	1.0	1.9	0.6	1.8
DLL3	0.9	1.6	1.7	1.3	0.6	0.5	15.6	3.5	2.6
DLL4	1.7	2.3	2.4	0.6	0.8	0.3	1.3	1.5	1.7
Jagged1	1.0	1.7	1.4	0.8	0.7	1.3	6.3	9.4	3.6
Jagged2	0.6	1.7	0.1	0.5	5.1	2.9	23.6	10.5	4.3
Notch 1	3.1	13.9	1.3	1.2	1.0	0.9	2.0	1.2	0.8
Notch2	0.4	0.1	0.3	0.3	0.3	1.8	1.8	0.8	1.1
Notch3	0.5	8.1	1.0	2.6	2.6	0.5	2.4	1.3	1.2
Notch4	0.5	3.3	0.4	1.0	0.8	0.5	1.9	4.2	1.2
Hes1	1.0	0.5	0.6	1.0	2.0	1.9	4.4	7.2	2.9

<2	1	>2

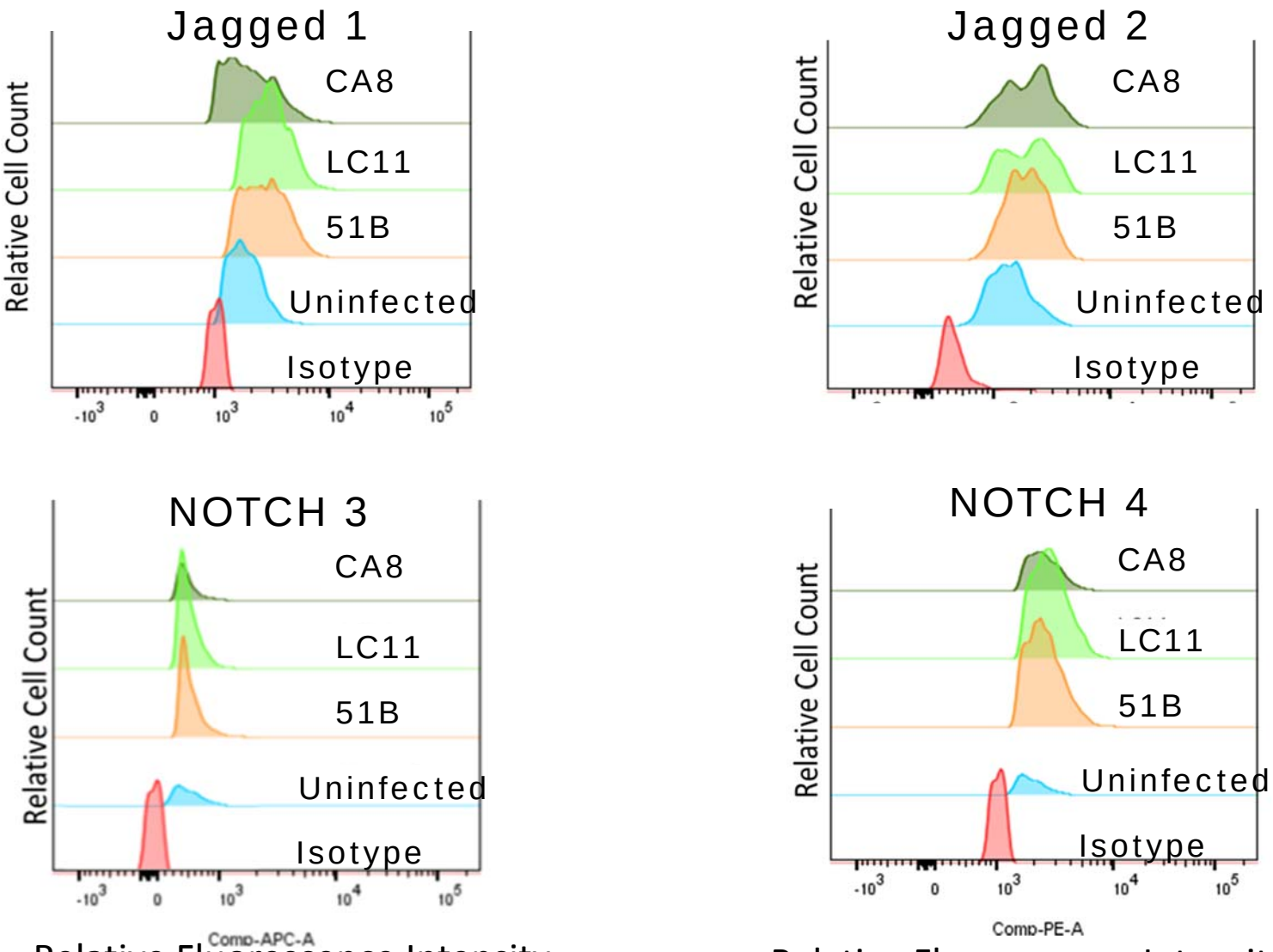


Figure 5. Flow cytometric analysis of AGS cell line infected with *Hp* isolates stained with antibodies to receptors and ligands of NOTCH signaling.